

ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN BLADDER PAIN SYNDROME/ INTERSTITIAL CYSTITIS

ANA JÚLIA DOS REIS PEREIRA

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ANA JÚLIA DOS REIS PEREIRA

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Orientador(a): Doutora Ana Cristina Estrela de Oliveira Charrua Cordeiro
Categoria: Bolseira de Pós-Doutoramento (Investigadora)
Afiliação: Faculdade de Medicina da Universidade do Porto
Co-orientador(a): Professora Doutora Célia da Conceição Duarte Cruz
Categoria: Professora Auxiliar
Afiliação: Faculdade de Medicina da Universidade do Porto

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List of Abbreviations and Symbols

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α_1 -adrenergic – Alpha₁-Adrenergic
Akt/PKB – Protein Kinase B
Bax – Bcl-2-associated X protein
BPS/IC – Bladder Pain Syndrome/ Interstitial Cystitis
 Ca^{2+} – Calcium cation 2+
Caspase 3 – Cysteine-Aspartic-Acid-Protease Member 3
DAB – diaminobenzidine tetrahydrochloride hydrate
ELISA – Enzyme-Linked Immunosorbent Assay
PHE – Phenylephrine
NGF – Nerve Growth Factor
NO – Nitric Oxide
NRP(s) – Neuropilin(s)
NRP2 – Neuropilin 2
PBS – Phosphate Buffer Saline
PBST – Phosphate Buffer Saline + Triton X-100
PKC – Protein Kinase C
PLC γ – Phospholipase C gamma
PIGF – Placental Growth Factor
PIGF-IR – Placental Growth Factor Immunoreaction
VEGF – Vascular Endothelial Growth Factor
VEGFA – Vascular Endothelial Growth Factor Member A
VEGFA120 – Vascular Endothelial Growth Factor Member A Isoform 120
VEGFA121 – Vascular Endothelial Growth Factor Member A Isoform 121
VEGFA145 – Vascular Endothelial Growth Factor Member A Isoform 145
VEGFA165 – Vascular Endothelial Growth Factor Member A Isoform 165
VEGFA189 – Vascular Endothelial Growth Factor Member A Isoform 189
VEGFA206 – Vascular Endothelial Growth Factor Member A Isoform 206
VEGFB – Vascular Endothelial Growth Factor Member B
VEGFC – Vascular Endothelial Growth Factor Member C
VEGFD – Vascular Endothelial Growth Factor Member D
VEGF-IR – Vascular Endothelial Growth Factor Immunoreaction
VEGFR1 – Vascular Endothelial Growth Factor Receptor 1
VEGFR1-IR – Vascular Endothelial Growth Factor Immunoreaction
VEGFR2 – Vascular Endothelial Growth Factor Receptor 2

VEGFR3 – Vascular Endothelial Growth Factor Receptor 3

VEGFRs – Vascular Endothelial Growth Factor Receptors

TRPV1 – Transient Receptor Potential Cation Channel

Abstract

Abstract

Bladder pain syndrome/Interstitial Cystitis (BPS/IC) is characterized by suprapubic pain related to bladder filling, usually accompanied by urgency and nocturia, in the absence of urinary infection or other pathology. Recently, it has been proposed a hypothesis that hyperactivity of the sympathetic nervous system may be associated with the altered pain sensation, bladder histological changes and/or urinary frequency associated with BPS/IC.

Vascular Endothelial Growth Factor family molecules and its receptors are overexpressed in the bladder wall of BPS/IC patients and in animal models. In this study, it is hypothesized that sympathetic dysfunction, induced by chronic administration of phenylephrine (PHE), may result in changes in VEGF expression and function in the urinary bladder.

The expression of Vascular Endothelial Growth Factor (VEGF), Placental Growth Factor (PlGF) and Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) in the urinary bladder (urothelium and detrusor muscle cells) was analysed by immunohistochemistry. It was observed basal expression of VEGF and PlGF in the urinary bladder (urothelium and detrusor muscle cells) from control animals. In PHE-treated rats, an increase of VEGF, PlGF and VEGFR1 expression was observed in areas of damaged urothelium, characterized by low urothelium.

VEGF expression and VEGFR1 in the detrusor muscle from treated rats was not altered, when compared to their expression in control animals. VEGF urinary levels were also determined by ELISA. The results obtained for concentration of VEGF in urine from PHE-treated rats and the one in urine from control rats were inconclusive.

We can speculate that VEGF and related molecules may have a relevant contribution to inflammatory and apoptotic events in chronic adrenergic stimulated animals.

Resumo

Resumo

O Síndrome Doloroso Vesical/ Cistite Intersticial é caracterizado por uma dor supra púbica relacionada com o enchimento da bexiga, normalmente acompanhada de frequência e noctúria, na ausência de infeção urinária ou outra patologia. Recentemente foi proposta a hipótese que a hiperatividade do sistema nervoso simpático possa estar associada com uma sensação de dor alterada, alterações histológicas na bexiga e/ ou aumento de frequência urinária associada à cistite intersticial.

As moléculas da família do VEGF e os seus recetores estão sobre expressos na parede da bexiga dos pacientes com cistite e em modelos animais. Neste estudo, foi colocada a hipótese da presença de disfunção do sistema nervoso simpático induzida por administração crónica de fenilefrina, o que poderá resultar em alterações na expressão e na função do VEGF na bexiga.

A expressão do VEGF, PIGF e VEGFR1 na bexiga (urotélíio e células do músculo detrusor) foi então analisada por imunohistoquímica. Observou-se expressão basal do VEGF e PIGF na bexiga (urotélíio e células do músculo detrusor) dos animais controlo. Nos ratos tratados com fenilefrina, um aumento da expressão de VEGF, PIGF e VEGFR1 foi observada em áreas de urotélíio degradado, caracterizado por um urotélíio baixo. A expressão de VEGF e VEGFR1 no músculo detrusor dos animais tratados não se alterou em relação à expressão das mesmas nos animais controlo.

Os níveis de VEGF na urina foram determinados por ELISA. Os resultados obtidos tanto para a concentração de VEGF na urina dos ratos tratados com fenilefrina e como para a concentração da mesma na urina dos ratos controlo foram inconclusivos.

Pode-se especular que o VEGF e moléculas relacionadas podem ter uma contribuição relevante em eventos inflamatórios e apoptóticos em animais sob estimulação crónica adrenérgica.

Introduction

Introduction

Bladder Pain Syndrome/Interstitial Cystitis (BPS/IC)

Bladder Pain Syndrome/Interstitial Cystitis (BPS/IC) is a chronic pathology that affects millions of people worldwide, being more prevalent in women with the mean age of 40 (Lau & Bengtson, 2010). This pathology is characterized by suprapubic and pelvic pain related to bladder filling usually accompanied by urinary frequency (in most severe cases, patients can urinate between 25 to 60 times per day), urgency and nocturia, in the absence of urinary infection or other cause (Cheppudira *et al.*, 2008; Temml *et al.*, 2007). Thus, the common hallmark of BPS/IC is pain without a readily demonstrable pathology of the viscera or associated nerves (Birder *et al.*, 2011). This has a negative impact in quality of life, by decreasing work productivity and sexual intimacy (Nickel *et al.*, 2007). The etiology and pathophysiology of BPS/IC is not known yet (Cheppudira *et al.*, 2008), and there is no reliable biological marker or effective therapy (Saban *et al.*, 2008a). Several hypotheses have been proposed, such as urothelium dysfunction, a defect of the glycosaminoglycan layer, infection by unknown bacteria, autoimmune mechanisms and neurogenic inflammation (Hanno *et al.*, 2010). Several reports demonstrate that patients with BPS/IC have increased levels of nitric oxide (NO), which can augment bladder wall permeability to water and urea, causing ultrastructural modifications in the apical layer (Birder *et al.*, 2011). Furthermore, urothelial cells from BPS/IC patients present altered patterns of expression of cell survival and adhesion genes (Keay *et al.*, 2012; Zhang & Kong, 2005).

In addition, there is no consensus regarding the diagnostic criteria for BPS/IC. Patients are selected according to the perception of pelvic pain and pressure related to the urinary bladder (Van de Merwe *et al.*, 2008). The exclusion of confusable diseases is made by analysing the past medical history, physical examination, analysis to urine and urine cultures, prostate-specific antigen in males above 40 years old, uroflowmetry, post-void residual urine volume by ultrasound scanning (Van de Merwe *et al.*, 2008). Hydrodystension at cystoscopy under anaesthesia and, in some cases, biopsies are also recommended (Van de Merwe *et al.*, 2008). Histopathological examination of bladder biopsies from classic common BPS/IC patients shows extensive inflammation, and alterations of urothelium or bladder epithelium thinning mucosal lesions (Gamper *et al.*, 2009; Birder *et al.*, 2011). Inflammation signs include detrusor mastocytosis, a mast cell counts exceeding 28 mast cells per mm² (Van de Merwe *et al.*, 2008). Mast cells may also play a central role in the pathogenesis and in pathophysiology of BPS/IC (Gamper *et al.*,

2009). They migrate into tissue perivascular spaces, and upon activation secrete granule-stored or de novo-synthesized molecules that mediate inflammatory reactions (Gamper *et al.*, 2009). Mucosa lesions may consist of glomerulations or Hunner's lesions (**Figure 1**). Glomerulations are characterized by bleeding at cystoscopy under hydrodistension, in which grade 2 is defined as large submucosal bleeding (ecchymosis) and grade 3 shows diffuse global mucosal bleeding (Gamper *et al.*, 2009; van de Merwe *et al.*, 2008). Hunner's lesions are described as a distinctive inflammatory lesion showing a particular deep rupture through the mucosa and submucosa, caused by bladder distension (Van de Merwe *et al.*, 2008). Alteration of microvessel structure, indicated by pericyte coverage, might be important in the formation of the glomerulations (Kiuchi *et al.*, 2009). It is already known that angiogenesis and angiogenic components are important in many chronic inflammatory diseases. (Kiuchi *et al.*, 2009) Actual evidence sets VEGF signaling in the centre of molecular pathways underlying urinary tract inflammation (Saban *et al.*, 2008b). Curiously, molecules of vascular endothelial growth factor (VEGF) family are involved in angiogenesis control, in the recruitment of inflammatory cells and in the alteration of urothelium histology (Cheppudira *et al.*, 2008; Kiuchi *et al.*, 2009; Saban *et al.*, 2008a)

	Cystoscopy with hydrodistention			
	Not done	Normal	Glomerulations ^a	Hunner's lesion ^b
Biopsy				
Not done	XX	1X	2X	3X
Normal	XA	1A	2A	3A
Inconclusive	XB	1B	2B	3B
Positive ^c	XC	1C	2C	3C

^a Cystoscopy: glomerulations grade 2-3.
^b With or without glomerulations.
^c Histology showing inflammatory infiltrates and/or detrusor mastocytosis and/or granulation tissue and/or intrafascicular fibrosis.

Figure 1. Classification of types of bladder pain syndrome considering analysis of the findings of cystoscopy with hydrodistention and of biopsies (from Van de Merwe *et al.*, 2008; EU 53: 60)

VEGF family and their receptors

The VEGF family is composed by five members: Vascular Endothelial Growth Factor Member A (VEGFA), Vascular Endothelial Growth Factor Member B (VEGFB), Vascular Endothelial Growth Factor Member C (VEGFC), Vascular Endothelial Growth Factor Member D (VEGFD) and placental growth factor (PIGF) (Suto *et al.*, 2005).

The isoforms of the VEGF family with different biological activities are derived from the alternative splicing (Olsson *et al.*, 2006). The human isoforms are denoted Vascular Endothelial Growth Factor Member A isoform 121 (VEGFA121), Vascular Endothelial Growth Factor Member A isoform 145 (VEGFA145), Vascular Endothelial Growth Factor Member A isoform 165 (VEGFA165), Vascular Endothelial Growth Factor Member A isoform 189 (VEGFA189) and Vascular Endothelial Growth Factor Member A isoform 206

(VEGFA206) (Olsson *et al.*, 2006). The mouse isoforms are one amino-acid residue shorter than the corresponding human isoform, and they are denoted Vascular Endothelial Growth Factor Member A isoform 120 (VEGFA120) and so forth (Olsson *et al.*, 2006). The bioactivity of VEGF family members can be regulated by proteolytic processing (Olsson *et al.*, 2006). This event might lead to specific interactions with distinct kinds of receptors (Olsson *et al.*, 2006).

VEGFA is known for its angiogenic and potent vascular permeability-enhancing activity (Cheppudira *et al.*, 2008). It supports and enhances the growth and regeneration of nerve fibers, with combination with other angiogenic, neurotrophic and neuroprotective molecules (Saban *et al.*, 2011). In fact, dysregulation of angiogenic factors have been associated to neurological disorders, especially neurodegenerative pathologies (Saban *et al.*, 2011). Therefore, VEGF is thought to be implicated in the cross-talk between nerves and vessels (Saban *et al.*, 2011).

PIGF is involved in pathological angiogenesis acting at different levels, such as directly stimulating the growth, migration and survival of endothelial cells (chemotactic action) leading to vessel growth and maturation, by increasing the proliferation and recruitment of smooth-muscle cells and supporting the proliferation of fibroblasts (De Falco, 2012). In addition, PIGF is essential for the recruitment and maturation of bone marrow-derived progenitors involved in angiogenic process and to promote differentiation and activation of monocyte-macrophage lineage that are able to further support the angiogenic stimulus (De Falco, 2012). Therefore, it can increase vascular permeability and survival *in vivo* (De Falco, 2012). In many cases, it can act by increasing the VEGF-induced effect, promoting the response of endothelial cells to VEGF in a cell-type-specific and time-dependent manner (Mac Gabhann & Popel, 2004).

The different VEGF isoforms exert their actions upon binding the three already described forms of VEGF receptors: VEGF receptor 1 (VEGFR1), VEGF receptor 2 (VEGFR2) and the rare VEGF receptor 3 (VEGFR3) (Olsson *et al.*, 2006) (**Figure 2**). VEGF (A and B) and PIGF bind both VEGFR1, VEGFA binds VEGFR2, and VEGFC and VEGFD bind VEGFR3 (Mac Gabhann & Popel, 2004). VEGFR1 is involved in chemotaxis of inflammatory cells and a negative regulator of VEGFR2 signalling capacity (Takahashi & Shibuya, 2005). This negative regulation is partially accomplished through the binding of a VEGFR1 soluble splice variant that binds to VEGF and thereby prevents VEGF from binding to VEGFR2 (Olsson *et al.*, 2006).

VEGFR2 has a role both in normal or pathological vascular-endothelial-cell biology, and VEGFR3 is relevant for lymphatic-endothelial-cell development and function (Olsson *et al.*, 2006).

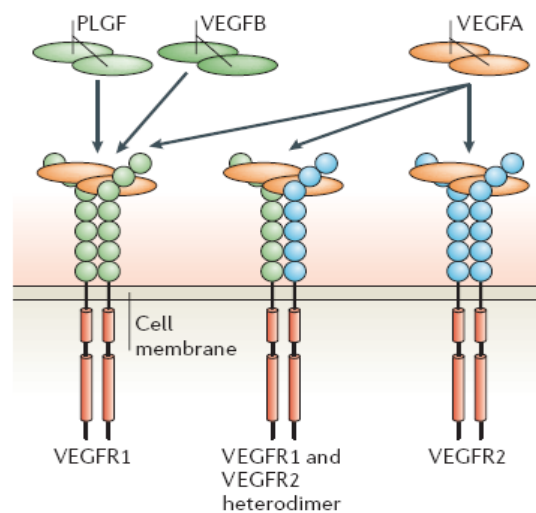


Figure 2: Localization of VEGF receptors 1 and 2 homodimeric and heterodimeric structures and respective ligands (Olsson *et al.*, 2006)

VEGFA and its splice variants activity vary according to their different abilities to interact with VEGFR co-receptors (neuropilins and heparan sulphate proteoglycans) (Lee, *et al.*, 2005; Woolard *et al.*, 2004). In human bladder urothelium, mounting evidence suggests a clear abnormality in the distribution of VEGF-Rs and neuropilins (NRP), which might be important in pathophysiology and diagnosis of BPS/IC (Saban *et al.*, 2008a).

VEGF molecules and their receptors in BPS/IC

VEGF expression is related to the modifications that might occur in the bladder vascular system (Saban *et al.*, 2011). VEGF has a strong expression in the urothelium, as well as a slightest expression in endothelial cells. In lamina propria, VEGF expression is mild (Kiuchi *et al.*, 2009). VEGF receptors are functionally active and localised in urothelial and neuronal cells in the urinary bladder, suggesting potential physiological functions of VEGF signalling in these cells (Saban *et al.*, 2008b). Human umbrella cells have an intense expression of VEGFR1 and NRP2, contrasting with their poor expression in the lamina propria and the detrusor muscle (Saban *et al.*, 2008a). In these last two layers, they seem confined both to blood vessels and inflammatory cells (Saban *et al.*, 2008a). It has been hypothesized that the VEGF receptors expressed in non-endothelial cells are involved in mechanism of cell survival (Saban *et al.*, 2008b).

In BPS/IC some of the above mentioned proteins expression changes drastically. VEGF levels are increased in lamina propria and diffuse in the extracellular space of

BPS/IC patients that present glomerulations under hydrodistension (Kiuchi *et al.*, 2009; Saban *et al.*, 2011; Saban *et al.*, 2008a). In addition, experiments using animal models of urinary bladder inflammation demonstrated that VEGF expression increases in the bladder wall (Saban *et al.*, 2011). Increased expression of VEGF during cystitis is involved in nerve plasticity (Saban *et al.*, 2011). In fact, VEGF induces an increase in substance P and TRPV1 expression, two molecules known to be expressed in urinary bladder nociceptors (Saban *et al.*, 2011), indicating an additional role in the control of pain pathway. PIGF expression and role in BPS/IC is unknown.

VEGFR1 and NRP2 expression is decreased during BPS/IC (Saban *et al.*, 2008a). Since VEGF molecules and their receptors have a role in cell survival in normal conditions, it has been hypothesized that the observed changes in the expression of these molecules represent an additional layer of protection for urothelium (Saban *et al.*, 2008a). However, if in the urothelium they present a similar role to the one observed in the control of vascular permeability and angiogenesis, they can be involved in urothelial leakage observed in BPS/IC (Saban *et al.*, 2008a).

Recent observations seem to indicate that there is a linkage between sympathetic dysfunction and VEGF molecules and their receptor activity. Overactivity of sympathetic nervous system may lead to degeneration of the bladder tissue, loss of bladder cells, angiogenesis and inflammation (Birder *et al.*, 2011; Gamper *et al.*, 2009; Kiuchi *et al.*, 2009). Increased sympathetic activity results in an increased noradrenaline release (Stein *et al.*, 1999) which has apoptotic effects on urothelial cells, promote bladder inflammation and sensitize bladder nociceptors leading to bladder hyperactivity and increased noxious input (Charrua *et al.*, 2011). In fact, increased sympathetic activity, neuron density, and vasomotor tone have been demonstrated in bladder tissues from patients suffering from BPS/IC (Stein *et al.*, 1999).

The main goal of the present work is to understand if there is a relation between sympathetic dysfunction and VEGF molecules and their receptors expression. In order to achieve this goal the bladder of chronic adrenergic stimulated rats was analysed for the expression of VEGF, PIGF and VEGFR1, by performing immunohistochemistry for these proteins and Enzyme-linked Immunoenzymatic Assay (ELISA).

Materials and Methods

Materials and Methods

Animals

Twelve female Wistar rats (Charles River Laboratories, Barcelona, Spain) with an average weight of 275g were used. Animals were maintained in the animal house at 22 °C and 60% humidity under a 12-hour light-dark cycle. All experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Animal model

Animals were divided in two groups of six animals each: control group and treated group. Animals from control group received subcutaneous injection of saline solution and treated group received subcutaneous injection of 2.5 mg/Kg phenylephrine, both daily for 14 days. At day 15, animals were anaesthetized with sodium pentobarbital 20% and the urine was collected to two tubes, one for each group of animals, and stored at -20°C until further use. The urinary bladders were harvested and fixed in 10% formalin, overnight at 4°C. Then they were dehydrated, included in paraffin and sectioned at 5 µm.

Immunohistochemistry and Image acquisition

Bladder sections were deparaffinised and rehydrated (xylol, ethanol 100%, ethanol 90%, ethanol 75% and distilled water, 5 minutes each). Antigen retrieval was achieved by heating the bladder slides immersed in 3g/L citrate buffer pH=6.0, in microwave oven, in maximum potency until boiling (around 7 minutes), then boiled for one minute, and then up to 15 minutes in the lowest potency. Slides were let in citrate buffer at room temperature until complete cooling. Then they were washed in 0.1 M Phosphate Buffer Saline (PBS) (10 minutes), and endogenous peroxidase was blocked in 1% hydroxide peroxide in methanol (v/v), for 10 minutes. After washing once more with 0.1 M PBS (10 minutes), the tissue was blocked with a solution of 10% normal horse serum in 0.1 M Phosphate Buffer Saline + Triton X-100 (PBST). Three sections of each animal were incubate with each of the following primary antibody, at 4°C, two overnights: 1:50 rabbit anti-VEGF (sc-152 (A-20) Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1:100 goat anti-PIGF (sc-27135 (K-20), Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA),

1:50 rabbit anti-VEGFR1 (RB-1527-P, Neomarkers, Fremont, CA). After three washes with 2% normal swine serum in PBST, for 10 minutes each, tissues were incubated with a 1:200 swine anti-rabbit secondary antibody (Dako, Glostrup, Denmark) and 1:200 HRP conjugate donkey anti-goat (catalogue no. sc-2020, Santa Cruz Biotechnology Inc.), for 1 hour at room temperature. The secondary antibody was washed twice with 2% normal swine serum in PBST, for 10 minutes, and once with PBST, for 10 minutes. Tissues were then treated with 1:200 horseradish peroxidase combined with biotin and avidin (VECTASTAIN® Elite ABC Reagent), for 1 hour, at room temperature. After washing with 0.1 M PBS (5 minutes) and with 0.1 M Tris-HCl (5 minutes), the tissue was incubated with 1.4 mM 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) + 1: 4000 hydrogen peroxide in a 0.05 M Tris-HCl solution. DAB solution was washed with 0.1 M Tris-HCl (5 minutes), 0.1 M PBS (5 minutes) and distilled water (5 minutes), and then counterstained with Hematoxylin. Bladder sections were then dehydrated (distilled water, ethanol 70%, ethanol 90%, ethanol 100%, xylol, each for 5 minutes) and mounted using Entellan Neu. All tissues were visualized with a Zeiss Vision Axioskop 40 photomicroscope and imaged using a AxioCam MRc5 camera driven by Axion Vision Rel 4.8 software. Immunopositive staining was evaluated by measuring the optical density on an arbitrary scale between 0—white pixels and 255—black pixels in three urothelial or detrusor areas per bladder section with National Institutes of Health ImageJ 1.43u software. The average optical density in each section was then calculated.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) kit was used to quantify urinary VEGF. Urine from control (n=6) and PHE-treated rats (n=6) were assayed as described below, using RayBio® Rat VEGF ELISA Kit. Briefly, 96-well plates were coated with anti-VEGF polyclonal antibodies. Urine or VEGF standards were added to each well, and plates were incubated. After several washes, anti-VEGF monoclonal antibodies were added, and plates were again incubated. After thorough washes, the amount of bound antibody was detected using immunoglobulin horseradish peroxidase-streptavidin conjugate antibody. The unbound conjugate was washed out, followed by 30 minute incubation with tetramethylbenzidine, at room temperature. Stop solution was added to terminate the reactions. Colour change was measured with a microplate reader TECAN infinite® 200 No. 30017581 (Mannëdorf, Switzerland) at 450 nm. All samples were run to duplicate, and values were averaged. VEGF levels were normalized to the urinary concentration of creatinine.

Creatinine Assay

The same two pools of urine used for VEGF quantification were assayed for creatinine quantification, according to the specified protocol and using Creatinine (urinary) Assay Kit (Item No. 500701, Cayman Chemical Company, USA). Sample or Creatinine standard were added to the respective wells. After incubation with alkaline picrate for 10 min, the absorbance was read at 495 nm (initial absorbance). After 20 min of incubation with acid solution, absorbance was read at 495 nm (final absorbance). The difference between the initial and the final absorbance reading of each standard, followed by the correction of the values subtracting the background value, generated the adjusted creatinine standard curve. The sample creatinine concentration was determined using the adjusted creatinine standard curve, previously subtracting initial and final absorbance readings and, to the total of this difference, subtracting the background value.

Statistical Analysis

Results are presented as the mean \pm standard deviation. Mean differences were compared by one-way ANOVA, followed by the post hoc Bonferroni test for multiple comparisons using SigmaStat® 2.03 software with $p < 0.001$ considered statistically significant.

Results

Results

Expression of VEGF in the urothelium

In the current work, we aimed at observing the expression of VEGF in the urothelium from control and PHE-treated rats. This animal model was employed to characterize the influence of chronic adrenergic stimulation in the urinary bladder, more specifically the urothelium, considering the expression of VEGF and related molecules. An initial assessment of VEGF levels was performed using immunohistochemistry.

Intensity signal of VEGF immunoreaction (VEGF-IR) in the urothelium of the urinary bladder of control animals was 109 ± 47 (**Figure 3A and 3D**). In PHE-treated animals, the signal intensity of VEGF-IR was 86 ± 61 in normal urothelium (**Figure 3B and 3D**) and 311 ± 180 in regions with low urothelium (**Figure 3C and 3D**).

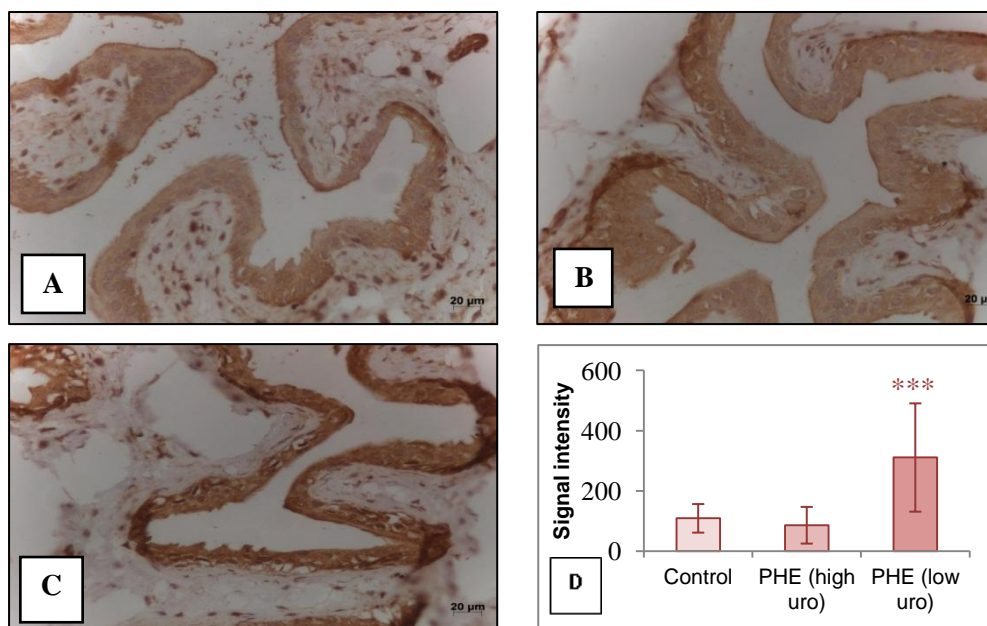


Figure 3: Photomicrographs of VEGF expression in the urothelium of control rats (A) and in high (B) and low urothelium (C) from PHE 2.5 mg/Kg -treated rats. D: Mean signal intensity of VEGF expression in the urothelium of a control and in the normal urothelium areas (high uro) and low urothelium areas (low uro) of PHE 2.5 mg/Kg treated rats. (***) $P < 0.001$

Taken together, these data showed an increase in VEGF expression in low urothelium from PHE-treated rats, when compared to high urothelium from PHE-treated and the one of control rats.

Expression of PIGF in the urothelium

PIGF belongs to the VEGF family, and expression might be altered due to the chronic adrenergic stimulation.

In this experience, we aimed at studying the expression of PIGF, in the urothelium from control and PHE-treated rats. PIGF levels were analysed by immunohistochemistry.

Intensity signal of PIGF immunoreaction (PIGF-IR) in the urinary bladder of control animals was 45 ± 22 (**Figure 4A and 4C**). In PHE-treated animals, the signal intensity of PIGF-IR was 64 ± 11 in normal urothelium (**Figure 4B, black arrows, and 4C**) and 370 ± 248 in regions with low urothelium (**Figure 4B, red arrow, and 4C**).

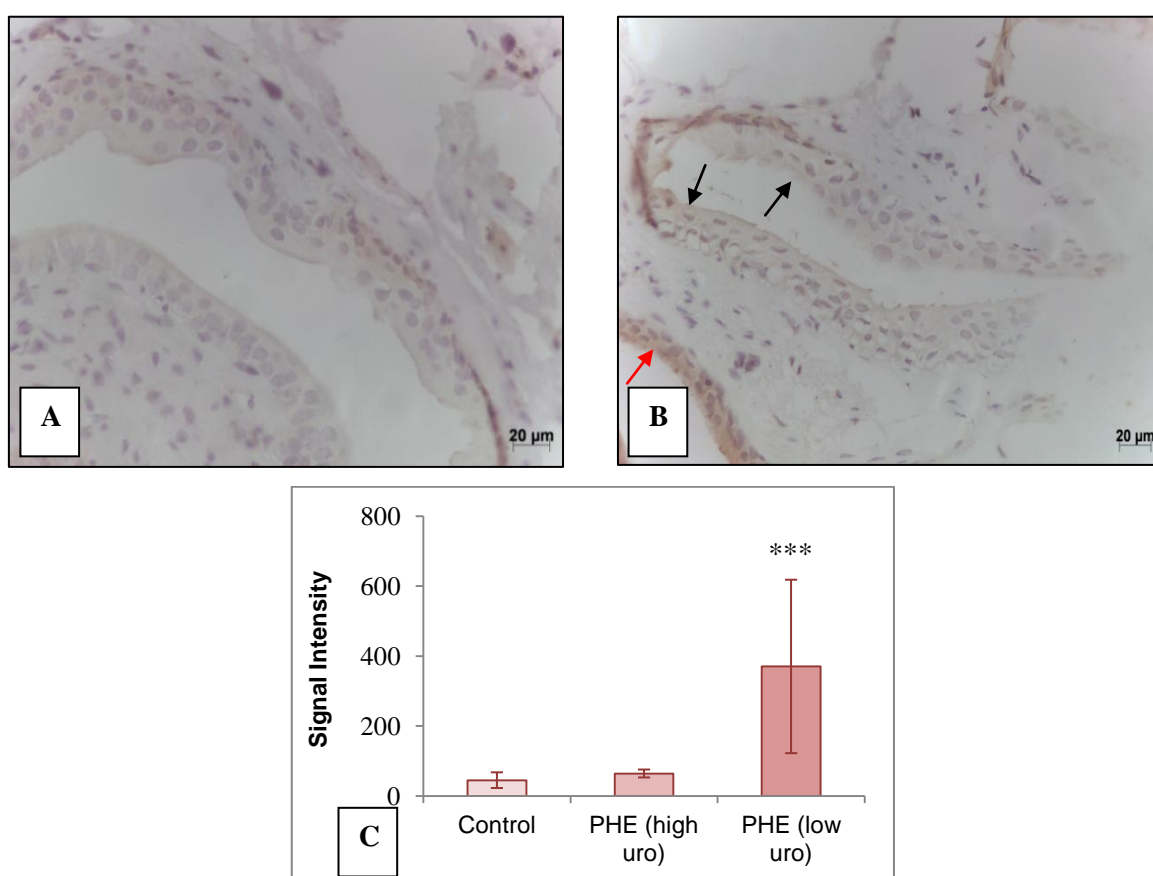


Figure 4: Photomicrographs of PIGF expression in the urothelium of control rats (A) and in high (B, dark arrows) and low urothelium (B, red arrow) from PHE 2.5 mg/Kg -treated rats. C: Mean signal intensity of PIGF expression in the urothelium of a control and in the normal urothelium areas (high uro) and low urothelium areas (low uro) of PHE 2.5 mg/Kg treated rats. (***) $P < 0.001$

Together, these data show an augment in PIGF expression in low urothelium from PHE-treated rats, when compared to high urothelium of PHE-treated rats and the urothelium from control rats.

Expression of VEGFR1 in the urothelium

In this experience, we intended to analyse the expression of the receptor for VEGF and PIGF, VEGFR1, in the urothelium from control and PHE-treated rats. VEGFR1 levels were determined by immunohistochemistry.

Intensity signal of VEGFR1 immunoreaction (VEGFR1-IR) in the urinary bladder of control animals was 102 ± 4 (**Figure 5A and 5D**). In PHE-treated animals, the signal intensity of VEGFR1-IR was 107 ± 5 in normal urothelium (**Figure 5B and 5D**) and 128 ± 9 in regions with low urothelium (**Figure 5B and 5D**).

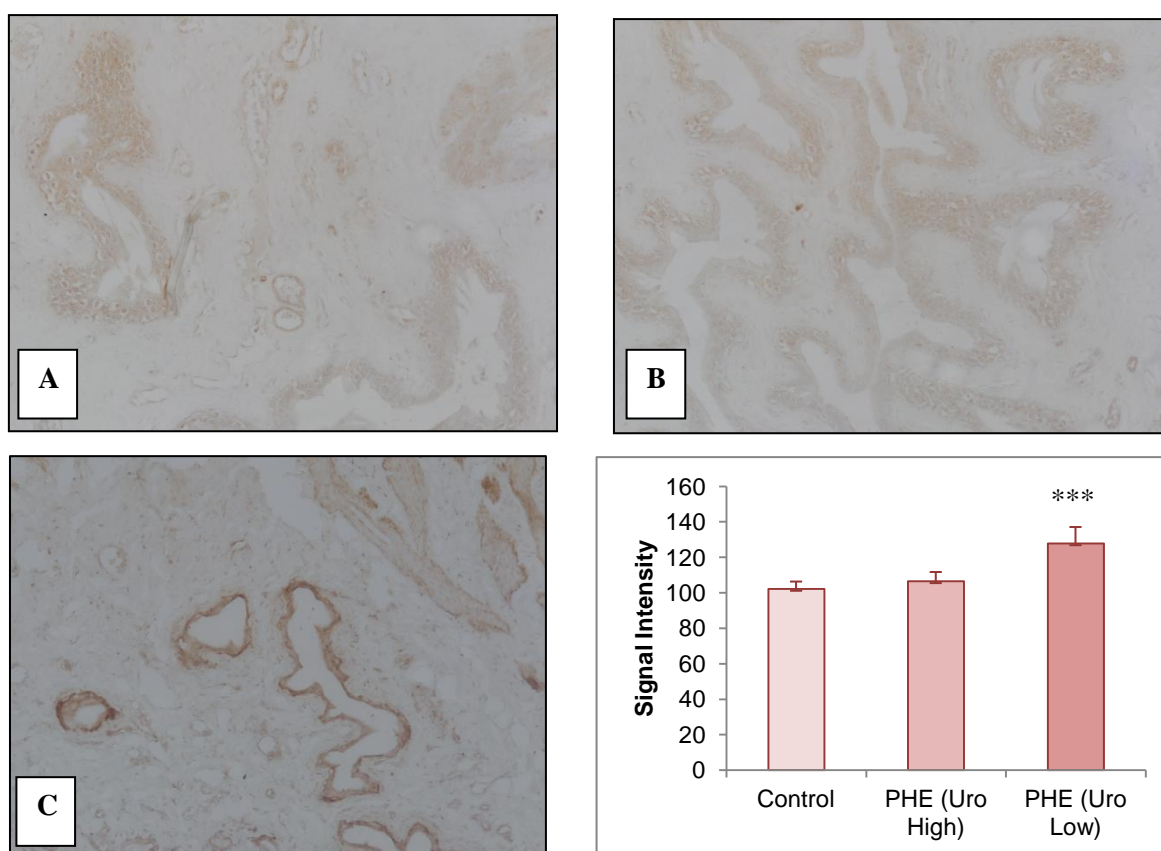


Figure 5: Photomicrographs of VEGFR1 expression in the urothelium of control rats (A) and in high (B) and low urothelium (C) from PHE 2.5 mg/Kg -treated rats. D: Mean signal intensity of VEGFR1 expression in the urothelium of a control and in the normal urothelium areas (high uro) and low urothelium areas (low uro) of PHE 2.5 mg/Kg treated rats. (***) $P < 0.001$

VEGFR1 expression was higher in low urothelium from PHE-treated rats, when compared to high urothelium of PHE-treated rats and the urothelium from control rats.

Expression of VEGF in the detrusor muscle

In this experience, we aimed at observing the expression of VEGF in the detrusor smooth muscle from control and PHE-treated rats. VEGF levels were analysed by immunohistochemistry.

Intensity signal of VEGF immunoreaction (VEGF-IR) in the detrusor muscle of the urinary bladder of control animals was 79 ± 9 (**Figure 6A and 6C**). In PHE-treated animals, the signal intensity of VEGF-IR was 82 ± 10 in the detrusor muscle (**Figure 6B and 6C**).

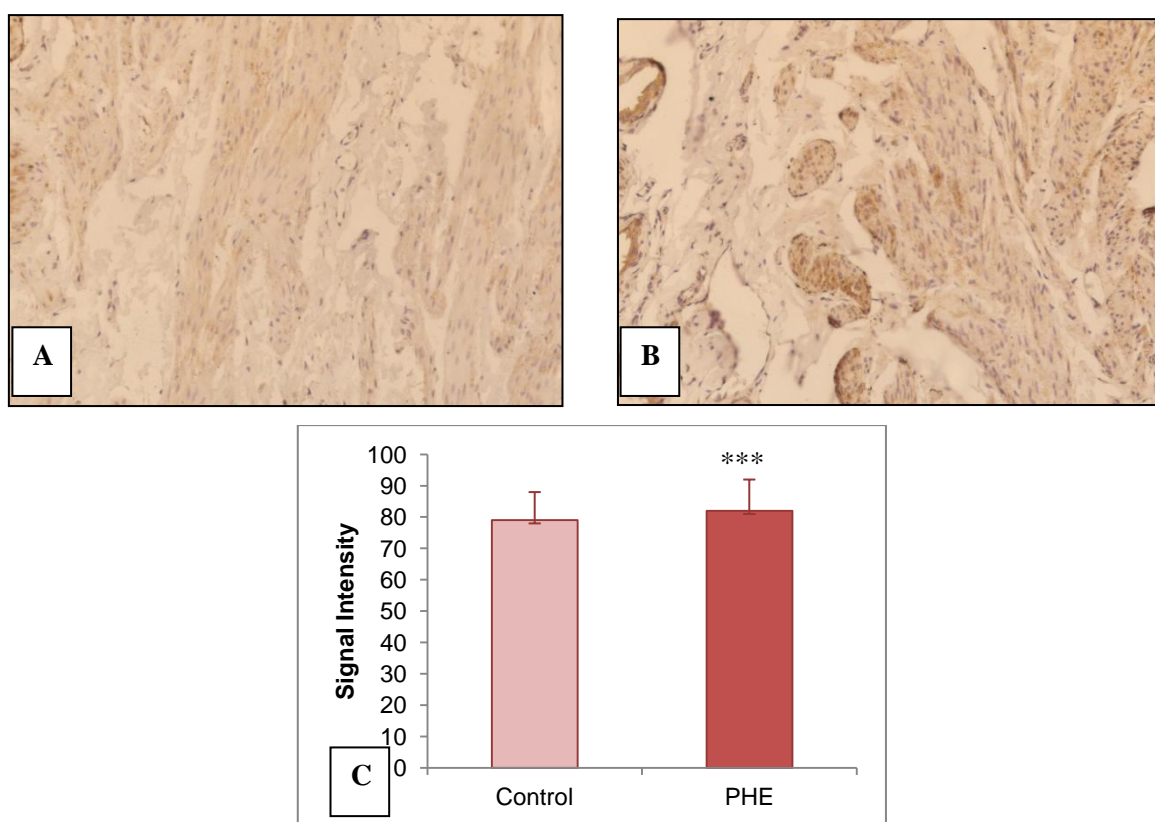


Figure 6: Photomicrographs of VEGF expression in the detrusor muscle from control (A) and PHE 2.5 mg/Kg -treated rats (B). C: Mean signal intensity of VEGF expression in the detrusor muscle from a control and PHE 2.5 mg/Kg treated rats. (***) $P < 0.001$)

No difference was observed between detrusor from control and from PHE-treated rats.

Expression of VEGFR1 in the detrusor muscle

In this experience, we intended to observe the expression of VEGFR1 in the detrusor smooth muscle from control and PHE-treated rats by immunohistochemistry. Intensity signal of VEGFR1 immunoreaction (VEGFR1-IR) in the detrusor muscle of the urinary bladder of control animals was 96 ± 5 (**Figure 7A and 7C**). In PHE-treated animals, the signal intensity of VEGFR1-IR was 97 ± 6 in the detrusor muscle (**Figure 7B and 7C**).

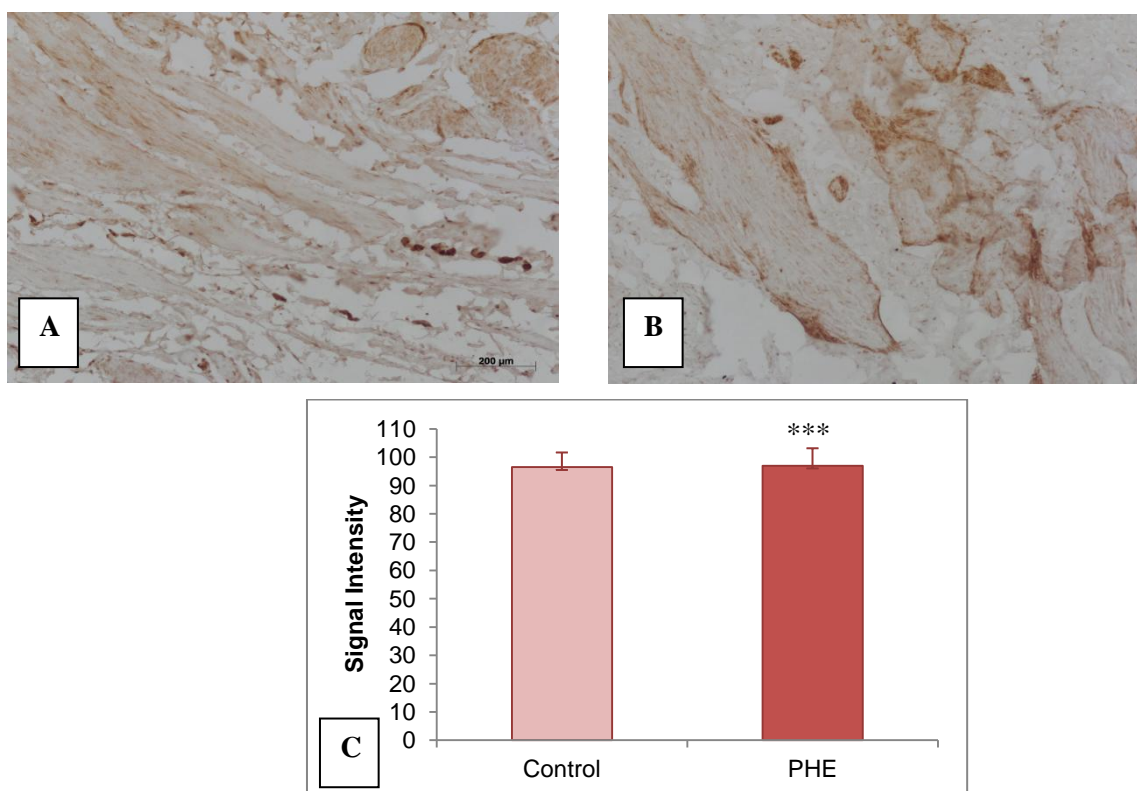


Figure 7: Photomicrographs of VEGFR1 expression in the detrusor muscle of control (A) and PHE 2.5 mg/Kg -treated rats. (B). C: Mean signal intensity of VEGFR1 expression in the detrusor muscle from a control and PHE 2.5 mg/Kg treated rats. (***) $P < 0.001$)

Taken together, these data show no difference between VEGFR1 expression observed in detrusor muscle from PHE-treated and from control rats.

VEGF quantification in urine

In this experience, we intended to quantify VEGF levels in urine from control and PHE-treated rats recurring to ELISA. The urine of control rat presented a VEGF concentration of 104.0 pg/mL, creatinine value of 2265,6 nmol/mL and 0.05 pg VEGF/nmol creatinine. The urine from PHE-treated rats presented VEGF concentration of 62.6 pg/mL, creatinine value of 2575.7 nmol/mL and 0.02 pg VEGF/nmol creatinine.

Chart 1: Values of final concentration VEGF, creatinine and ration pg VEGF/nmol creatinine in the urine from control and PHE-treated rats.

Samples	Final concentration VEGF/ (pg/mL)	creat_u/ (nmol/mL)	pg VEGF/ nmol creatinine
control	103.96	2265.58	0.05
PHE	62.59	2575.72	0.02

The value obtained for VEGF concentration in urine from PHE-treated rats and the one obtained in urine from control rats were inconclusive.

Discussion

Discussion

VEGF, PIGF and VEGFR1 expression upon chronic adrenergic stimulation in the urothelium

In the present work we aimed to understand if there is a relation between sympathetic dysfunction and VEGF molecules and their receptors expression. In order to achieve this goal the bladder of chronic adrenergic stimulated rats was analysed for the expression of VEGF, PIGF and VEGFR1.

Chronic adrenergic stimulation induced the disappearance of umbrella cells in some areas of the bladder. From now on these areas will be entitled of low urothelium and the areas with all the urothelium layers will be named high urothelium. Immunostaining against VEGF revealed that the high urothelium of PHE-treated rats and control animals had similar expression pattern. However, the low urothelium from chronic adrenergic treated animals had a higher expression of VEGF. This increase in the expression of VEGF can be related with PHE-induced mast cell migration to the mucosa (Charrua *et al.*, 2011). In fact, it has been shown that VEGF is involved in proliferation, migration and recruitment of inflammatory cells leading to persistent chronic inflammation (Cheppudira *et al.*, 2008). VEGF is a chemoattractive molecule synthesized by urothelial cells (Cheppudira *et al.*, 2008). In fact, VEGF binding to mast cells VEGFR1 could promote activation/migration of these cells, and therefore have a role in inflammation. (Cheppudira *et al.*, 2008; Ciurea *et al.*, 2011; Shibuya, 2006)

Furthermore, it has been shown that VEGF is involved in induction of apoptosis (Narasimhan, *et al.*, 2009). Overstimulation of α_1 adrenergic receptor would probably lead to the hypertonicity of the urothelial cells, due to the constant contraction of the cells (Raz & Rodriguez, 2008) The levels of Ca^{2+} , derived from the α_1 adrenergic receptor activation by PHE, may increase in the cytosol and trigger a cascade of intracellular changes and activity (Luo *et al.*, 2007). VEGFA binding to VEGFR1 may induce apoptosis through a PLC γ -PKC dependent mechanism (Nishizuka, 1995; Olsson *et al.*, 2006). This could explain the decrease in urothelial height and the expression of pro-apoptotic caspase 3 in the more external urothelial layers of PHE-treated animals (Charrua *et al.*, 2011). VEGF increased levels in the urothelium, upon chronic adrenergic stimulation, reflect the observed in BPS/IC patients. (Kiuchi *et al.*, 2009)

The expression of PIGF in the urothelium of control and PHE-treated animals was similar to the VEGF expression. PIGF is also involved in proliferation, migration and

recruitment of inflammatory cells possibly leading to persistent chronic inflammation (De Falco, 2012). VEGF and PlGF might have a synergic effect on inflammation in the urothelium. In fact, it has been shown that PlGF can increase the response to VEGF in animal models of endothelial cell survival, migration and proliferation, acting directly or competing for the receptor (Mac Gabhann & Popel, 2004).

VEGFR1 has similar expression both in urothelium from control and PHE-treated rats. However, overexpression of this protein was observed in low urothelium, similarly to what happens to its ligands. This result suggests that VEGF and PlGF may have an autocrine activity in urothelial cells. Hence, under chronic adrenergic stimulation, degradation of urothelium may upregulate VEGFR1 expression, in order to enhance the VEGF and PlGF effects, such as apoptotic activity (Tsao *et al.*, 2004).

Interestingly, VEGFR1 expression is downregulated in the urothelium from BPS/IC patients (Saban *et al.*, 2008a). This difference might be explained due to the fact that BPS/IC bladder specimens are obtained after hydrodistension, and therefore submitted to mechanical stimuli (Saban *et al.*, 2008a).

VEGF, PlGF and VEGFR1 expression upon chronic adrenergic stimulation in detrusor smooth muscle

Detrusor smooth muscle from control and PHE-treated animals expressed VEGF. Chronic adrenergic stimulation did not alter the expression of this molecule. This result is in accordance to the observed in detrusor smooth muscle of BPS/IC patients (Cheppudira *et al.*, 2008; Saban *et al.*, 2008a). In the urinary bladder of BPS/IC patients, there is a migration of mast cells, lymphocytes and plasma cells chemotaxis into the detrusor muscle (or muscularis propria) (Gamper *et al.*, 2009; van de Merwe *et al.*, 2008). The present results and the ones observed in the literature seem to indicate that this migration is not directly mediated by VEGF molecules (Cheppudira *et al.*, 2008; Saban *et al.*, 2008a). However, in these animals, the existence of inflammatory events mediated by other pro-inflammatory molecules (e.g. Nerve Growth Factor (NGF), pro-inflammatory cytokines) cannot be excluded (Cheppudira *et al.*, 2008). Signals may be released from an abnormal urothelium or penetrating through damaged urothelium and may stimulate submucosal and muscular nerves, leading to the recruitment and activation of inflammatory leukocytes, including mast cells. These mast cells will release mediators that will maintain inflammation. (Gamper *et al.*, 2009)

VEGF quantification in urine

Urinary VEGF levels obtained for both of the pools of urine (PHE-treated rats and control rats) were inconclusive.

There were only two pools of urine (urine from control rats versus urine from treated rats). The volume of urine collected from each rat was reduced, so two pools were made from each group. One solution to make the results statistically significant is to collect more samples of urine from a major number of rats, control and PHE-treated rats, and organize them in more pools.

Conclusions

Conclusions

The present work showed that chronic treatment with phenylephrine in female rats caused the overexpression of VEGF, PIGF and VEGFR1 in the low bladder urothelium. One can hypothesize that VEGF and PIGF acting on VEGFR1 may induce urothelial degradation, by activating apoptotic enzymes.

Chronic adrenergic stimulation did not induce any change in VEGF, PIGF and VEGFR-1 expression in the detrusor smooth muscle, suggesting that VEGF related molecules are not involved in direct control of detrusor activity during chronic adrenergic stimulation.

The results obtained for VEGF concentration in urine from PHE-treated and control rats were inconclusive. A major number of samples should be assessed in order to understand if VEGF levels changes upon adrenergic stimulation.

Future Prospects

Future Prospects

Further approaches should be done in order to understand the true role of VEGF in BPS/IC. VEGF might be involved in inflammatory, angiogenic, apoptotic and chemotactic processes in BPS/IC. Previous studies in PHE-chronically treated rats showed the occurrence of apoptotic in the urothelium and bladder inflammation. The present study raises the hypothesis that VEGF related molecules may be involved in these mechanisms. Therefore, co-localization of VEGF and its related molecules and apoptotic enzymes should be addressed. Furthermore, experiment conduction to the confirmation of VEGF chemotaxis role should be addressed.

Although chronic adrenergic stimulation does not induce any macroscopic changes in the urinary bladder, such as petechial appearance, angiogenesis can be addressed by studying pericyte migration and new vessel forming. VEGF signalling should be studied at a molecular level in this model in order to fully understand the apoptotic and angiogenic events, for instance involvement of Akt/PKB or PKC, which may explain the apoptosis, and relation between VEGFR1 and VEGFR2.

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